EXHIBIT 21
PART 4

(Ea_c) and homologous rabbit antibody, as well as the cross reaction with duck egg albumin (Ea_d) (123).

Effect of Antibody Concentration

If the quantity of antigen is kept constant and antibody is allowed to vary, a C′ fixation curve is obtained which consists of two linear segments, the first with a large positive slope, and the second displaying a very small positive slope. The break between the two segments is quite sharp and occurs near the ratio of antibody to antigen which would be in or near the equivalence zone for the precipitin reaction of the same antigen-antibody system. A family of curves for the system pneumococcus polysaccharide SIII reacting with homologous rabbit antibody is shown in Figure 70 (123).

Effect of Variation in Concentration of Antigen-Antibody Complex, with Ratio of Reactants Kept Constant at Equivalence Zone Ratio

This has been studied by Wallace *et al.* (126) for the system bovine serum albumin-homologous rabbit antibody, and results are shown in Figure 98 in Chapter 7. It is noteworthy that the fixation of complement, as a function of the quantity of immune complex, follows a sigmoidal curve. This is due to the fact that the over-all hemolytic activity of complement, under the experimental conditions used in (126), depends primarily on the concentration of C′2 and C′3. C′1 and C′4 play a secondary role, as explained above. Hence, fixation of some C′1 and C′4 by a small amount of antigen-antibody complex would have little effect on the over-all titer. It is also of significance that the experiments in Figure 98 in Chapter 7 were conducted at 2-4°C., where C′3 is not fixed appreciably.

The significance of these findings with respect to heterogeneity of antibody is discussed in Chapter 7.

Speed of Complement Fixation

A representative experiment illustrated in Figure 71A shows that the speed of fixation of C′ is greatest at the beginning of the reaction and diminishes progressively. In studies at 37°C. a fixation period of

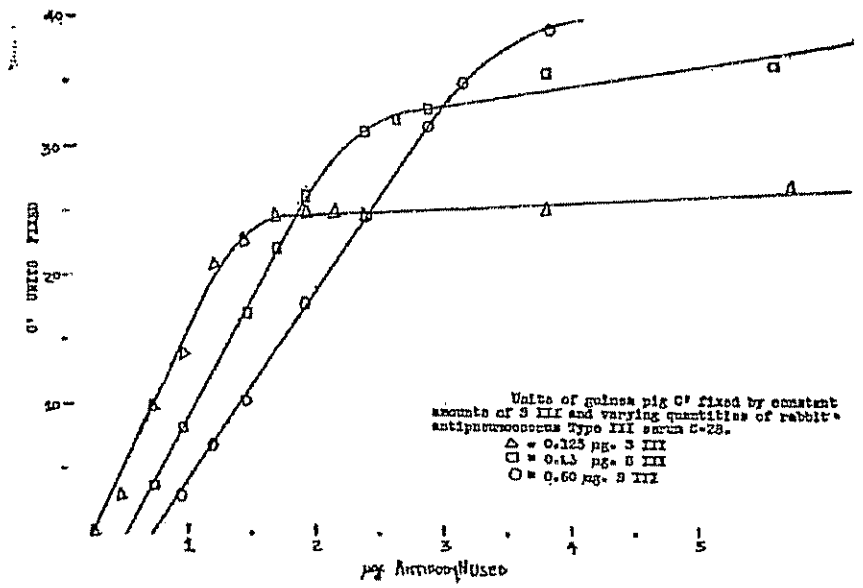

FIG. 70. Fixation of guinea pig complement by constant amounts of S III and varying quantities of rabbit antipneumococcus Type 3 serum. From (123).

ninety minutes has been employed usually, but this does not represent an endpoint. Indeed, there is no endpoint, but fixation of C′ continues to increase slowly with time. This is to be expected from the mechanism of fixation of the complement components as discussed below.

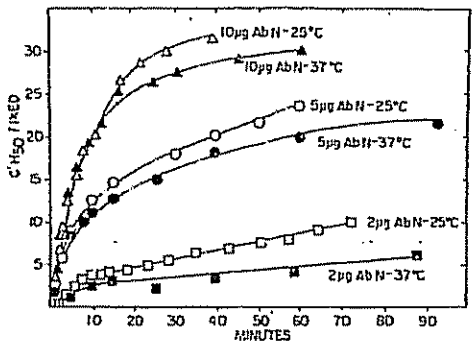

FIG. 71A. Kinetics of complement fixation at 25°C. and at 37°C. with rabbit antipneumococcus serum Type 3 and equivalent amounts of S III. From (127).

Effect of Temperature

As shown in Figure 69, the extent of C′ fixation is higher in a reaction run overnight in the cold than in ninety minutes at 37°C. The reason for this is not clear. C′1, C′4 and C′2 are fixed in the cold, but fixation of C′3 (measured by its action on EAC′1, 4, 2) is markedly dependent upon temperature. The temperature effect is greater in cross reactions than in homologous reactions (cf. Fig. 69).

Effect of C′ Concentration

C′ fixation experiments with 50, 100, and 200 $C'H_{50}$ are shown in Table 17. In reactions conducted at 37°C., fixation increased progressively as the total quantity of C′ was raised from 50 to 200 $C'H_{50}$. On the other hand, at 3°C. there was almost no difference between 100 and 200 $C'H_{50}$. Interpretation of these observations requires more information on the behavior

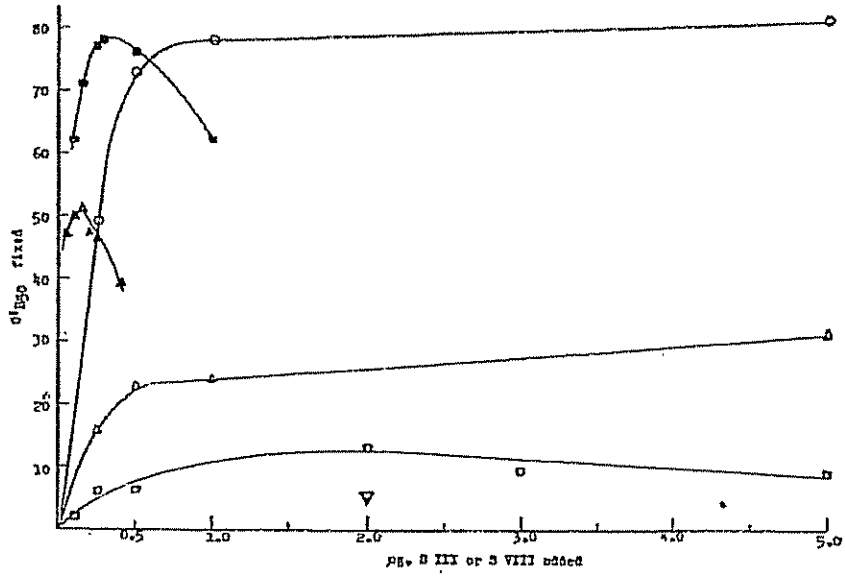

FIG. 71B. Fixation of C′ by varying quantities of S III and 2.0 μg of antibody N from rabbit anti-Pn Type III serum 819 at 37°C. (▲) and in the cold (■).
Fixation of C′ by varying quantities of S VIII and 2.6 μg (□), 5.2 μg (Δ), 10.4 μg (○) of cross-reacting antibody N from rabbit serum 819 in the cold.
Fixation of C′ by 2.0 μg of S VIII and 5.2 μg of cross reacting antibody N from rabbit serum 819 at 37°C. (▽) From (124).

TABLE 17

Comparison of $C'H_{50}$ Fixed by 1.9 μg Antibody N (Rabbit Antipneumococcus Type III Serum C-28) With Increasing Quantities of S III for 90 Min. at 37°C. and Overnight at 3°C.

Amount of S III added	50 $C'H_{50}$ added		100 $C'H_{50}$ added		200 $C'H_{50}$ added	
	Temp. of fixation		Temp. of fixation		Temp. of fixation	
	37°C.	3°C.*	37°C.	3°C.	37°C.	3°C.
μg.						
0.1	23		28	56	48	63
0.15	29		36	75	53	76
0.20	29		36	82	53	87
0.25	27		34	83	52	86
0.30	25		30	82	47	84
0.40	20		22	78	47	74
0.50	16		16	68	33	73

*Complete fixation occurred in several of the tubes, and very few units were left in the others. Similar data were obtained with rabbit antipneumococcus III serum 819 and S III. From (122).

of the C′ components than is available at present.

Fixation of the Components of C′

Available evidence indicates that fixation of the complement components by antigen-antibody complexes is entirely comparable to their reaction with sensitized erythrocytes. Hence, it can be assumed with reasonable confidence that antigen-antibody aggregates first react with C′1, if Ca^{++} is present, followed by reaction with C′4, C′2 and C′3. For example, Osler *et al.* (12) prepared GAC′1, 4, 2 complexes (G = antigen, A = antibody) which reacted with C′3. Similarly, Ishizaka has made GAC′1, 4 complexes by decay from GAC′1, 4, 2, and the reaction of these decayed complexes with C′2 has been investigated. In studies with the reagents R1, R2, R3 and R4 there has been general agreement that C′1, C′4 and C′2 are fixed by antigen-antibody aggregates. With respect to fixation of C′3 there has been controversy. It is probable that the discordant observations with respect to the fixation of C′3 have arisen from differences in experimental conditions, notably, differences in temperature and in the concentration of C′ in the reaction system. It has been noted already that the fixation of C′3 is favored at body temperature and by the use of a high concentration of C′. This is a matter of importance in view of the fact that the recognition and definition of properdin rests on inactivation of C′3 by the postulated zymosan-properdin complex. It is implied in this definition that the inactivation of C′3 is an exclusive property of zymosan-properdin complexes or of complexes of properdin with certain other polysaccharides, such as, inulin or the lipopolysaccharides from *Salmonella typhi*. It should be noted that inactivation of C′3 by these complexes is performed at 37°C. with a concentration of complement which is considerably greater than that customarily used in C′ fixation reactions. In view of the demonstration by Nelson (88) that antigen-antibody complexes fix C′3, under appropriate experimental conditions, and the finding by Osler *et al.* (12) that fixation of C′3 by antigen-antibody complexes at 37°C. is quite extensive, it is no longer possible to maintain the concept that inactivation of C′3 is a special and exclusive attribute of complexes formed by "properdin" with certain polysaccharides. As pointed out in a later section on "properdin," this invalidates the basic definition applied by

Pillemer's group, and casts serious doubt on the existence of "properdin" as an entity distinct from natural antibody.

Complement Fixing Efficiency of Antibody

It has been generally assumed, though tacitly, that the complement fixing titer of an antiserum is a measure of its antibody content, but this is not strictly true. It has been shown by Wallace *et al.* (126), as illustrated in Figure 98 in Chapter 7 that rabbit antisera to bovine serum albumin may vary in their capacity to fix complement when compared at equal levels of antibody weight, reacting with equivalent concentrations of antigen. It is evident from this figure that the quantity of anti-BSA required for fixation of 50 out of 100 $C'H_{50}$ varied from about 1 to 4 μg of nitrogen. The variations observed by Wallace *et al.* were correlated with differences in immunization schedules, i.e., antisera from animals which have received two courses of inoculations fixed more complement per unit antibody weight than antisera from animals given only one course of immunization. In view of these findings, the complement fixing titer of antiserum should be regarded as a reflection of *both* the quantity and the quality of its antibody.

This matter has been studied further by Hill and Osler (127, 128) who found a correlation between the rate of flocculation of antiserum in the reaction with antigen, and the C′ fixing potency per unit of antibody weight. Furthermore, partial absorption of an antiserum revealed a marked degree of heterogeneity with respect to the specific C′ fixing potencies of different portions of the antibody in a single serum. It was concluded that the C′ fixing efficacy of antibody depends on its affinity for the antigen, as judged by aggregating capacity.

This concept would be in accord with the hypothesis of Heidelberger (22) that molecules of antibody form a loose association with C′, and that C′ becomes firmly bound when several molecules of antibody are brought into close proximity as a result of aggregation through interaction with antigen. Ishizaka's observations (47) that heat-aggregated human γ-globulin fixes C′ is also in accord with this concept.

Some antigen-antibody systems do not fix complement or do so only by special techniques. Notably, certain avian or bovine antisera, if heat-inactivated, do not fix guinea pig complement well and hence an indirect method is used (see below). Although some horse antibody systems fix complement (e g., anti-typhoid O antigen), horse antisera to pneumococcus type-specific capsular polysaccharide do not (171), unless the method of quantitative C′ fixation analysis is used (172). Human antisera to pneumococcal capsular polysaccharide have shown variable behavior (37, 173). Similarly, rabbit anti-carbohydrate damaged by acid does not fix C′ (174). The customary heat-inactivation of antiserum may produce damage which impairs C′ fixing capacity (cf. 175).

Estimation of Antigen by C′ Fixation

As shown in Figures 68 and 69, the curves which describe the course of C′ fixation by a constant level of antibody reacting with increasing amounts of antigen, resemble the corresponding curves observed in quantitative precipitin analyses. At ratios of the immune reactants corresponding to large antibody excess, the fixation of C′ is highly responsive to minute changes in antigen, thus providing a sensitive method for estimation of antigen. This approach has been used by Peterkofsky, Levine and Brown (176), for estimation of heat-labile alpha-2 glycoprotein in human serum. These investigators also presented data on the estimation of serum albumin in human sera by quantitative C′ fixation analysis and showed that results for this protein agreed with those obtained by electrophoresis within 10%.

The use of C′ fixation analysis for quantitative estimation of antigens yields results at a level of precision approaching that of quantitative precipitin analyses, with the advantage that far less material is consumed and the procedure is less laborious and more rapid than analyses of specific precipitates.

Cross Reactions

The cross reactions between pneumococcus polysaccharides Types 3 and 8 (Fig. 71B), and between hen and duck ovalbumins (Fig. 69) have been studied by Osler and Heidelberger (124, 125). It was found that the specific complement fixing potency per unit weight of cross reacting antibody was far lower than that for the homologous reaction. Different cross-reactive antisera varied widely in this respect. It is evident from these observations that the endpoint dilution titer of an antiserum in a cross reaction, as obtained by C′ fixation, does not furnish a reliable index of its antibody content. It was also noted in these studies that with some heterologous immune reactants a much greater excess of the cross-reacting than of the homologous antigen was required to inhibit the fixation of C′, in analogy to similar findings in quantitative precipitin studies. This means that in titrations of an antiserum with different but related antigens, a cross-reacting antigen may actually yield a higher serum titer than the homologous antigen, if relatively high concentrations of the antigens are used in the titrations. Therefore, in comparative titrations of this kind it is desirable to employ several different concentrations of each antigen, in order to avoid this possibility of error.

Quantitative C′ fixation analysis has also been used by Osler and Knipp (177) in studies of the Wassermann antibody in the sera of humans with syphilis, in sera of the "biological false positive" type and in rabbit antisera to cardiolipin. Beef heart, human heart and human liver phospholipids were found to be immunologically equivalent in these tests.

Levine *et al.* (178) applied the method in an investigation of low density plasma lipoproteins which cross react extensively. It was found that these lipoproteins represent a family of macromolecules, the members of which are closely related with respect to immunologic specificity, although they may be differentiated by ultracentrifugation.

The C′ Fixation Technic of Stein and Van Ngu (179)

This is a quantitative C′ fixation technic designed for high sensitivity. In essence, it resembles the quantitative C′ fixation analysis, except that only 2 $C'H_{50}$, instead of 50 or 100 are used. This level of C′ produces about 90% hemolysis, and, therefore, *any* degree of C′ fixation will be evident by a decrease of hemolysis below this level. It is not necessary to make dilutions of the reaction mixture, as in the quantitative C′ fixation analysis, but sensitized cells are added directly to the reaction mixture at the end of the fixation period. As the quantitative C′ fixation analysis, the method of Stein and Van Ngu furnishes precise measurement of the degree of anticomplementary action, if any, by antigen or antiserum alone.

The Method of Fulton and Dumbell (180)

This is a micro-technic designed for economy of material and rapidity of execution. For these reasons it has found use in virology and epidemiology. Instead of test tubes, Plexiglass plates are used. Reagents are delivered by drop from a calibrated tube or syringe and needle. A low concentration of red cells is employed and the cells are heavily sensitized with antibody so as to produce agglutination in those reaction mixtures which do not lyse. Thus, in a positive C′ fixation reaction the red cells are seen clumped in the center of the well.

In the use of this technic considerable care must be exercised to hold volumetric error to a minimum. Furthermore, evaporation of water from the reaction mixtures must be guarded against since even a small increase in ionic strength of the reaction medium exerts a pronounced effect on the hemolytic activity of C′. In addition, one should be aware of the changes in 1/*n* of the von Krogh equation, reflecting changes in the shape of the response curve, which are known to occur on variation of cell concentration and degree of sensitization of the cells with antibody. Pike *et al.* (181) have applied this technic in the study of leptospiral antigens. Black and Melnick (182) have modified it for use in poliovirus studies.

Indirect Complement Fixation Test

Certain mammalian and avian sera fix guinea pig complement poorly, or not at all in the usual C′ fixation test (183). This is probably not due to damage to the antibody as a result of heat-inactivation. Rice (184) studied this failure in the case of certain chicken, turkey, duck, and goose sera against *S. pullorum*, and found that these sera possessed the capacity to inhibit the C′ fixing activity of rabbit, guinea pig or pigeon antisera to *S. pullorum*. She designed an indirect or inhibitory C′ fixation test which has found wide application in veterinary medicine (185), for example, for the detection of ornithosis in chickens, ducks, turkeys and geese, or in foot-and-mouth disease of cattle. In essence, this is an antigen binding test, in which, for example, a chicken serum to *S. pullorum* is treated with pullorum antigen and complement, then a rabbit antiserum is added, and after further incubation sensitized red cells are introduced. If the chicken serum contains antibody, the antigen will be bound and complement fixation by the rabbit antibody, added subsequently, does not occur. Hence, a positive test is one giving lysis of the sensitized erythrocytes. Schmidt and Harding (186) have studied similar effects with certain human sera and lymphogranuloma venereum, psittacosis, mumps, Q-fever and lymphocytic choriomeningitis antigens.

Brumfield and Pomeroy (175) have reported that *unheated*, fresh chicken and turkey sera give *direct* complement fixation with ornithosis, Newcastle disease and infectious bronchitis antigens. This suggests that chicken and turkey antibody cannot fix guinea pig complement due to species incompatibility, unless a heat-labile factor from the antisera is present. A similar mechanism may apply to cattle sera. Marucci succeeded in detecting foot-and-mouth disease antibody in heat-inactivated cattle sera by *direct* complement fixation with vesicular fluid as antigen (187, 188); previous attempts with infected tongue epithelium as antigen had failed. Possibly, the postulated heat-labile, normal factor was supplied by vesicular fluid.

Method of Maltaner (49-53, 189, 190)

This is a technic in which the degree of fixation is measured quantitatively in terms of per cent hemolysis, instead of by the usual crude method in which results are recorded as +, ++, +++ or ++++.

In general, the method, as used by Maltaner *et al.* in routine tests for syphilis (49), involves the use of a quotient (IS + A)/IS where (IS + A) is the activity of the complement expressed in 50% units after incubation with immune serum (IS) plus antigen (A), and IS represents the activity after incubation with immune serum alone. After a preliminary titration to determine the 50% unit of the complement, tests are set up with one unit of complement and a fixed quantity of immune serum, and with 3, 6 and 12 units of complement and immune serum plus 3 doses of antigen so adjusted as to give the maximum reaction in at least one combination with sera of low, moderate and marked activity, respectively. If the immune serum is of moderate potency, the

test with 3 units may show no lysis while the 6 unit test may be in the partial range of hemolysis. In case of a strong antiserum both 3 and 6 unit tests may exhibit no lysis but the 12 unit tube may be partially lysed. The degree of hemolysis in the partly hemolyzed tube is read and, using conversion factors such as those in Table 3, the amount of complement is found which would have given 50% lysis. For example, if the 6 unit tube shows 30% hemolysis, 6.0/0.844 = 7.1 units would have given 50% hemolysis. The degree of hemolysis in the 1 unit tube incubated with serum alone is also read and converted by application with the proper factor into the amount of complement which would have given 50% lysis. For example, 1 unit of complement in the presence of immune serum may produce 20% lysis. Then 1.0/0.758 = 1.32 units would have given 50% lysis. The ratio of the amount of complement required for 50% lysis in the presence of immune serum and antigen to that required for 50% lysis in the presence of serum alone, is considered by Wadsworth *et al.* (52) to be an index of the potency of the immune serum. In this case the ratio would be 7.1/1.32 = 5.4. Quotients greater than unity are considered evidence of an immune reaction. Titers determined by this method may, however, be affected by the so-called fixability of the complement. Rice (191) points out that variations due to this factor may be overcome by including titrations with a standard serum so that the titer may be expressed in terms of the quotient: $\frac{\text{test (IS + A)}}{\text{test IS}} \Big/ \frac{\text{standard (IS + A)}}{\text{standard IS}}$. If the test sera as well as the standard sera are neither hemolytic nor anticomplementary, the quotient may be simplified to the form: test (IS + A)/standard (IS + A).

The relations between the amounts of complement fixed and of antibody and antigen in the Wassermann reaction have been extensively studied by Wadsworth and Maltaner (51). They were found to be linear when conditions were adjusted to maximal sensitivity. Linear relationships between serum and complement have also been obtained by Rice with gonococcal sera and antigen (192). The same author also demonstrated linearity between the amounts of rabbit antipneumococcus antibody and complement when maximally reactive quantities of antigen were employed (191). Similar relations have also been observed with tuberculous immune serum and antigen (50).

Certain aspects of complement fixation have been reviewed recently by Osler (24).

EXPERIMENTAL PROCEDURES FOR COMPLEMENT FIXATION

Titration of Antigen or Antibody by Complement Fixation

Pooled guinea pig serum distributed in test tubes or glass ampoules and kept frozen at −40°C. serves as a source of complement. Determine the hemolytic activity of each pool by titration of the contents of one or two ampoules according to the quantitative photometric procedure described in an earlier section. If all the test tubes or ampoules into which a pool of guinea pig serum has been distributed were treated uniformly it can be assumed that their contents have the same hemolytic complement titer. Therefore, it is not necessary to perform a complement titration before each complement fixation test. However, as explained below, a simple and approximate control titration is included with each complement fixation test.

While the spectrophotometric titration of complement is performed in a total reaction volume of 7.5 ml., the complement fixation tests are set up on one-fifth this scale, i.e., with a final hemolytic reaction volume of 1.5 ml., and results are read by visual inspection. For purposes of complement fixation the complement unit is also

scaled down by a factor of 5. For example, with a sample of guinea pig complement containing 200 $C'H_{50}$ per ml., as determined in the photometric titration with 5 x 10^8 optimally sensitized cells in a total reaction volume of 7.5 ml., 0.2 ml. of a 1/200 dilution added to 1 x 10^8 sensitized cells in a total reaction volume of 1.5 ml will yield 50% lysis.

Appropriate dilutions of antiserum and antigen for the complement fixation test are prepared so that 0.4 ml. of each will contain the desired quantity. Make a dilution of guinea pig serum so that 0.5 ml. will contain 5 $C'H_{50}$ (this refers to the one-fifth scale system). Since guinea pig serum usually contains about 200-250 $C'H_{50}$ per 0.2 ml. (one-fifth scale system), 0.5 ml. of a 1/100 or 1/125 dilution will usually furnish approximately 5 $C'H_{50}$. The actual dilution of guinea pig serum needed should be calculated from the hemolytic titer. The complement fixation test is set up by mixing 0.4 ml. of antiserum dilution, 0.5 ml. of guinea pig serum dilution as a source of complement, and 0.4 ml. of antigen dilution. This reaction mixture may be kept either for ninety minutes at 37°C. or for about twenty hours at 2 to 4°C. Overnight incubation in the cold has been found to be a more sensitive procedure. At the end of the incubation period, 0.2 ml. of a suspension of sensitized erythrocytes (5 x 10^8 cells per ml) is added, the contents are mixed, and the tubes are incubated at 37°C. for sixty minutes in a water bath with occasional agitation in order to maintain the cells in uniform suspension.

Since antiserum or antigen, or both, may "fix" or inactivate complement alone, i.e., non-specifically, it is necessary to include control tubes which contain antigen + complement, and antiserum + complement. In order to rule out non-specific reactions reliably, it is advisable to run these "anti-complementary" controls in a dual fashion, viz, with 5 $C'H_{50}$ and with 3 $C'H_{50}$. For the latter controls, use 0.3 ml. of the diluted guinea pig serum instead of 0.5 ml., and make up the difference in volume with 0.2 ml. of diluent (it is essential to maintain constant volume, since the hemolytic activity of complement depends on concentration).

It is also necessary to ascertain whether the antigen or antiserum contain any substance which hemolyzes erythrocytes non-specifically; i.e., without the benefit of complement. This kind of difficulty does not arise frequently but the lowest dilution of antiserum (highest concentration) and the lowest dilution of antigen should be checked for hemolytic activity in the *absence* of complement.

Before use, the antiserum should be heated for thirty minutes at 56°C. in order to inactivate its complement. Prepare the desired dilutions (it is usually not advisable to work with dilutions lower than 1/5; in some routine diagnostic tests in which patient's sera are tested within one or two days after collection, dilutions as low as ½ are used). In titrations of sera from patients or experimental animals the optimal quantity of antigen must be determined experimentally by testing a series of different dilutions, as in the protocol given below, and selecting the antigne dilution which yields the highest antiserum titer. For general guidance in complement fixation tests, the following protocol is presented:

		Amount and Dilution of Antiserum					Control	
		0.4 ml. 1/10	0.4 ml. 1/20	0.4 ml. 1/40	0.4 ml. 1/80	0.4 ml. 1/160	0.4 ml. Diluent	0.6 ml. Diluent
Volume and Dilution of Antigen	0.4 ml. 1/1000 0.4 ml. 1/2000 0.4 ml. 1/4000 0.4 ml. 1/8000 0.4 ml. 1/16,000 0.4 ml. Diluent	All tubes get 0.5 ml. C′ dilution containing 5 $C'H_{50}$						0.3 ml. C′ dilution
	0.6 ml. diluent	0.3 ml. C′ dilution						

Incubate either ninety minutes at 37°C. or twenty hours at 2-4°C. Then add 0.2 ml. sensitized cells to all tubes and incubate sixty minutes 37°C. Let settle and read by visual inspection. Record as follows:

0 = no lysis
1 = approximately 25% lysis
2 = approximately 50% lysis
3 = approximately 75% lysis
4 = approximately 100% lysis

Lytic Tests:

0.4 ml. 1/100 antiserum + 0.9 ml. diluent
0.4 ml. 1/1000 antigen + 0.9 ml. diluent

C′ Deterioration Test:

	Tube Number				
	1	2	3	4	5
C′ dilution containing 5 $C'H_{50}$ per 0.5 ml., ml. Veronal-saline, ml.	0.1 1.2	0.2 1.1	0.3 1.0	0.4 0.9	0.5 0.8
	Incubate with C′F test under chosen conditions				
Sensitized cells, ml.	0.2	0.2	0.2	0.2	0.2
	Incubate 60 minutes at 37°C.				

In routine diagnostic tests it may be necessary to go as low as ½ dilution of serum. In order to reduce experimental manipulations in this type of test, it is convenient to use 0.2 ml. of undiluted serum, instead of 0.4 ml. of a ½ dilution. This is done in the routine diagnostic procedure recommended by Osler and Strauss (193) for which a detailed protocol is given below:

Tube	1	2	3	4	5	6	7	8	9	10	11
Serum, undiluted, ml.	0.2	0.1	0.2	0.1	0	0	0	0	0	0	0
Veronal buffer, ml.	0	0.1	0.5	0.6	0.2	0.35	0.45	0.7	0.85	0.95	1.2
5 $C'H_{50}$ per 0.5 ml.	0.5	0.5	0.5	0.5	0.5	0.35	0.25	0.5	0.35	0.25	0
Antigen, ml.	0.5	0.5	0	0	0.5	0.5	0.5	0	0	0	0
Fixation: 20 hours at 3–5°C. + 20 minutes at 37°C.											
Sensitized erythrocytes, ml.	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Lysis: 30 minutes at 37°C.											

It should be noted that in this procedure only thirty minutes at 37°C. are allotted to the hemolytic reaction. Although this is not quite enough to reach an endpoint, the difference between thirty and sixty minutes is not of appreciable significance, since the tests are read by visual inspection. Furthermore, in this procedure 0.3 ml. of a sensitized erythrocyte suspension is used, and accordingly, the concentration is adjusted so that this dose of 0.3 ml. will contain 1 x 10^8 cells (i.e., a 2/3 dilution of the usual cell suspension).

Tubes 2 and 4 are included in this procedure in order to minimize the need for repeating tests for sera that are anticomplementary in 0.2 ml. volume. Furthermore, the possibility of inhibition of C′ fixation due to excess antiserum is also reduced.

The degrees of lysis in the test and in the control tubes are estimated visually, and color standards for purposes of comparison may be prepared by mixing a standardized suspension of red cells and a solution of hemoglobin in such proportions as to simulate 0%, 25%, 50%, 75% and 100% lysis. Tubes showing 50% lysis, or less, are read best by comparing the clear supernates after the unlysed cells have settled, while those above 50% lysis can be read most readily with the unlysed cells kept in suspension.

QUANTITATIVE COMPLEMENT FIXATION ANALYSIS

As described in (122), these analyses are performed by letting the desired quantities of antigen and antibody react with a rather large quantity of complement (50, 100, 200 or more $C'H_{50}$) either for ninety minutes at 37°C. or for twenty hours at 2-4°C. At the end of the fixation period accurately measured aliquots of the reaction mixture are diluted appropriately and quantitative hemolytic complement titrations are performed by the photometric procedure described in an earlier section in order to determine the number of residual $C'H_{50}$. Appropriate antigen, antibody and diluent controls are included, and the difference between the residual $C'H_{50}$ in the reaction mixtures and in the controls represents the number of $C'H_{50}$ which have been fixed specifically.

In most studies in which this procedure has been employed a total volume of 10 ml. has been used for the fixation reaction. Thus, 2.5 ml. of an appropriate dilution of antiserum are mixed in the cold in 50 ml. wide-mouth Pyrex centrifuge tubes, with 5.0 ml. of a dilution of guinea pig serum containing the number of $C'H_{50}$ desired. Large tubes are used in order to facilitate mixing of reagents. After addition of complement, 2.5 ml. of an appropriate dilution

of the antigen are added with thorough mixing. The tubes are capped and incubated in a water bath at 37°C. ± 0.1° or kept in a refrigerator at 2-4°C. overnight. (In cross-reacting systems it is advisable to maintain accurate control of temperature; for fixation in the cold the tubes should be kept in a container of crushed ice which is placed in a refrigerator.) Following the indicated period of fixation the tubes are chilled in ice-water in order to retard any further fixation of complement. A portion of the contents of each tube is diluted with chilled isotonic veronal buffer to yield a dilution suitable for the estimation of the residual activity of C′ in terms of $C'H_{60}$. For example, if it is anticipated that of 50 $C'H_{60}$ added, twenty will have been fixed, the 10 ml. fixation mixture would then contain 30 $C'H_{60}$, or 1 $C'H_{60}$ in 1/3 ml. Hemolytic C′ activity in the range of partial lysis would then be determined by testing 3.0, 3.5 and 4.0 ml. portions of a 10-fold dilution of the reaction mixture with 1.0 ml. of sensitized cells (5×10^8 cells) and sufficient chilled isotonic buffer (in this instance 3.5, 3.0 and 2.5 ml.) to make a final volume of 7.5 ml. The contents of the tubes are mixed, the tubes are capped and incubated at 37°C. for sixty minutes. After centrifugation the supernates are analyzed photometrically for hemoglobin to determine the degree of lysis The activity in terms of $C'H_{60}$ is obtained by graphic evaluation or by calculation with the aid of the conversion factors given in Table 3. A typical protocol showing the detailed steps is presented in Table 15.

CONGLUTININ

Observations by Bordet and Gay (194), as well as Muir and Browning (195) on the clumping of guinea pig erythrocytes by fresh horse serum in the presence of heated bovine serum, a phenomenon termed "conglutination" to differentiate it from agglutination reactions without complement, led to the finding that the heated bovine serum supplies two essential factors, namely, a natural antibody to the erythrocyte and an aggregating substance termed "conglutinin." Some samples of bovine serum may not contain sufficient natural antibody to the erythrocytes, but in these cases the clumping action of the beef conglutinin can be demonstrated with erythrocytes sensitized by traces of rabbit antibody insufficient to cause agglutination alone. Conglutinin can be absorbed from beef serum by erythrocytes which have been so sensitized and treated with complement from horse serum or certain other sera. Thus, substances other than complement can be fixed by antigen-antibody aggregates (cf. rheumatoid factor, ref. 17).

A test similar in principle to the hemolytic complement fixation reaction was developed by Streng (196) in which disappearance of conglutinating complement activity serves as an index of antigen-antibody interaction. Interest in the conglutinating complement absorption test (CCAT) has been stimulated by Hole and Coombs (197, 198), who found that fixation of horse complement in the conglutination reaction might be more sensitive in certain immune systems, for example, sera of ponies convalescent from glanders, than the hemolytic complement fixation test with guinea pig complement. Christine Rice has studied various aspects of the conglutination reaction and of the conglutinating complement absorption test (199-202), but her studies did not indicate any marked advantage of CCAT over hemolytic complement fixation tests. In tests of bovine sera with *Brucella abortus* suspensions she found that CCAT was more sensitive, but a larger proportion of sera were anticomplementary. Of special interest are her studies with certain avian antisera which do not fix hemolytic guinea pig complement. Horse complement absorption tests by the conglutination technic

yielded negative results with chicken and turkey pullorum antisera, sera of chickens convalescent from infection with Newcastle Disease virus, and chicken antisera to influenza virus.

The conglutinating complement absorption test is performed with sheep erythrocytes treated with heated bovine serum which supplies both sheep red cell antibody and conglutinin. Fresh horse serum serves as a source of complement. Details of procedure have been described by Hole and Coombs (197), and several minor modifications, including the use of traces of sheep cell hemolysin for sensitization, have been introduced by Rice and Avery (199).

The agglutination of Rh^+ cells by Rh blocking antibody in the presence of bovine serum to provide a high protein concentration (203, 204) has been termed conglutination by Wiener (204). This should not be confused with conglutination as used above since the blocking antibody agglutinates equally well in high concentrations of bovine or human albumin or even polyvinylpyrollidone (cf. I, 3).

A plate technic for the conglutinating complement fixation test has been described by Bier *et al.* (205), for detection of antibodies against foot-and-mouth disease viruses in guinea pig hyperimmune sera, as well as for estimation of C-reactive protein in human sera. In essence, this technic represents an adaptation of the procedure of Fulton and Dumbell (180).

Leon (206) has shown that Ca^{++} is necessary for the conglutination reaction. Mg^{++} and Ba^{++} are without effect, but Sr^{++} can be substituted for Ca^{++}, though it is less effective. The reaction is reversible by $MgNa_2$-or $BaNa_2$-EDTA, and this reversal can be overcome by addition of Ca^{++}. Leon has also shown that zymosan inhibits the conglutinating activity of beef serum by way of formation of a complex of zymosan with conglutinin.

BACTERICIDAL AND BACTERIOLYTIC REACTIONS

In conjunction with antibody, or other sensitizers, complement will kill bacteria of certain genera, such as Vibrio, Salmonella, Shigella or Escherichia, by damaging the cell wall. Although these reactions have been studied extensively in view of their significance in immunity, at present there is no detailed information on formation of intermediate products and the properties of the individual reaction steps, but it is known that C′1, C′2, C′3 and C′4, as well as Mg^{++}, are necessary (10). In addition, it has been observed that reactivity is enhanced by lysozyme; as well as by certain other materials extractible from leukocytes and platelets (207-209).

Experimental measurements have been made mostly by the technique of bacteriologic plate count. For review and detailed description of methodology the reader is referred to the monograph by Maaløe (10).

A photometric growth assay for measuring bactericidal activity has been described by Muschel and Treffers (210-212). In comparison to the plate count method this technique has two main advantages, namely, titrations can be performed more rapidly and relatively high precision (± 10%) can be achieved with less labor than by the plate count procedure. Muschel and Treffers (211) have shown that the bactericidal reaction system of *S. typhosa*, guinea pig complement and rabbit or human antibody resembles in several respects the hemolytic reaction system. Mg^{++} enhances bactericidal activity, the bactericidal response curves resemble those in the hemolytic reaction, bactericidal antibody activity and complement exhibit a reciprocal relationship, and the amount of antibody required for killing of one bacterial cell is very small. As in the hemolytic reaction, only a minute fraction of the cell surface needs to be in combination with

antibody. Muschel *et al.* (213) have applied the photometric growth technic to studies of the specificity of bactericidal action of normal sera against *S. typhi*, and their results are of interest to the properdin problem.

A quantitative bacteriolytic technic has been developed by Amano *et al.* (214) in studies with *E. coli B*. According to these investigators, under the action of antibody and complement, as well as serum lysozyme, or a lysozyme-like substance normally present in guinea pig serum, *E. coli B* are converted to spheroplasts, which are coccoid cells with a damaged or altered cell wall. By addition of sodium desoxycholate to a concentration of 0.05%, the spheroplasts are lysed, while the bacteria remain intact, permitting their separation by centrifugation. Perchloric acid is added to the supernatant fluid to a final concentration of 2%, the mixture is heated at 70°C. for twenty minutes, and after centrifugation the nucleic acid content of the supernatant fluid is determined photometrically at 260 mμ.

Through use of this method, it was shown by Inoue *et al.* (215) that lysozyme is necessary for the conversion of bacterial cells to spheroplasts and that this enzyme acts on the cells only after treatment with antibody and complement. Normal serum contains sufficient lysozyme for spheroplast formation, but if lysozyme is removed from the serum containing complement as well as the antiserum, by treatment with bentonite, spheroplast formation is abolished. Addition of crystalline egg white lysozyme restored the bacteriolytic activity (i.e., spheroplast formation) of the antibody-complement system. Furthermore, lysozyme accelerated and enhanced the bactericidal activity. These observations may be interpreted by assuming that antibody and complement act on the cell wall so as to uncover the substrate for lysozyme.

Serious technical difficulty in bactericidal and bacteriolytic measurements arises frequently from inability to remove sensitizers normally present in the serum containing complement, even if large amounts of bacteria are used for absorption. According to Amano *et al.* (214) this can be overcome by treatment of the serum containing complement with zymosan, in addition to absorption with bacterial cell walls (cell walls were used by these investigators because intact bacteria release nucleic acid into the serum used for complement during absorption).

Another technical difficulty arises from bacterial growth during the bacteriolytic experiment. In order to avoid this, Amano *et al.* (214) performed their bacteriolytic experiments with bacteria killed by exposure to ultraviolet light.

Treponema Pallidum Immobilization Test (TPI)

This is the only bactericidal reaction which has found wide application in diagnostic immunology. Since its discovery in 1949 (216), it has become firmly established as a primary reference standard among the various serologic tests for the diagnosis of syphilis. Specifically, the TPI reaction has furnished a sound basis for recognizing "biologic false positive reactions" which are encountered quite frequently in Wassermann tests. In addition, the TPI test has been found of value in the diagnosis of certain forms of late syphilis (217).

The TPI test is performed by admixture of motile treponemes with the patient's serum to be tested for antibody, and with normal guinea pig serum as a source of complement. After incubation at 35°C. for about sixteen to eighteen hours under anaerobic conditions, the extent of bactericidal action is evaluated by microscopic count of remaining motile organisms. While simple in principle, technical execution of this test is difficult because of the fact that *Treponema pallidum* cannot be cultivated *in vitro*, but must be obtained from treponemal lesions, usually from infected rabbit testes Furthermore, *Treponema*

pallidum is a fastidious organism which can be kept alive *in vitro* only by use of special media and by great care in manipulation. The difficulties of maintaining the test organism in a motile state are especially serious because an unusually long period of time is required for the immobilization reaction. As shown by Seldeen (218), this is due to the fact that several hours are needed for sensitization by the syphilitic antibody. It is believed that *Treponema pallidum*, when freshly isolated from a syphilitic lesion may have a surface layer of non-antigenic material, possibly hyaluronic acid, which is presumably lost slowly on incubation. Antibody can combine with the treponemes only after this protective layer is gone, and, in turn, the complement system can begin its destructive work only after sensitization has taken place.

Another unusual feature of the TPI reaction is the fact that normal guinea pig serum does not contain sensitizing antibody for *Treponema pallidum*, and similarly, normal human serum is devoid of antibody or other sensitizers. Consequently, when treponemes are exposed to normal human and normal guinea pig serum as a control, no immobilization occurs. Thus, in the absence of treponemal infection, a clean-cut negative test is obtained. Technical details for performance of the TPI test are given in (216, 219).

Hemolysis by Unrelated Antigen-Antibody Systems (Passive Hemolysis)

It is well known that red cells will adsorb certain polysaccharide antigens and it was shown by Adler (220, cf. 220a), in continuation of studies by Fisher and Keogh (221), that erythrocytes from sheep, rabbit or man, which have absorbed *E. coli* or *S. typhi* antigens can be lysed by complement and the corresponding antibodies. Hemolysis of human erythrocytes coated with *E. coli* antigen by antibody and complement has been studied by Neter *et al.* (222). Bier *et al.* (223) have studied passive hemolysis in relation to complement-fixing ability of the antibody. Ingraham (224) coupled sheep erythrocytes with sulfanilic acid and obtained hemolysis by treatment with complement and antibody to the sulfanilic acid group. Similar studies have been made by Silverstein and Maltaner (225) with erythrocytes to which p-azophenyl-arsonate groups were attached.

It is interesting to speculate whether the phenomenon of passive hemolysis bears any relation to anaphylactic or allergic reactions, in the sense that these manifestations of hypersensitivity also result from the interaction of unrelated antigen-antibody systems with cells of the hosts (cf. 23, 226, 24).

Properdin (11, 87, 88, 110, 114, 206, 213, 227-248)

In studies on the nature of C′3 it was discovered by Pillemer (87) that inactivation of this component by zymosan does not proceed directly but involves several reaction steps. It was found that zymosan combines with a substance in human serum which was designated properdin (P), to form an intermediate product, PZ, which, in turn inactivates C′3. Formation of PZ requires complement components C′1, C′2, C′4, as well as Mg^{++}, and proceeds optimally at about 15°C. Inactivation of C′3 by PZ also requires complement and Mg^{++}, and proceeds optimally at 37°C. It was indicated that the properties of properdin differ from those usually associated with antibodies in several respects: 1) Properdin does not combine with zymosan below 10°C., 2) The reaction requires Mg^{++}, 3) Complement components C′1, C′2 and C′4 are required for combination of P with Z, 4) Formation of PZ requires ionic strength below 0.4 and pH 6.5 to 8.2 (87).

Furthermore, it was indicated that PZ inactivates C′3 selectively and only at temperatures above 20°C. (87), whereas

according to Pillemer (87), antigen-antibody complexes fix C′1, C′2 and C′4, but not C′3, either at 0°C. or 37°C. Treatment of human serum with zymosan below 20°C. removes properdin, but not C′3, and the resulting reagent lacking P is designated RP.

Properdin, Mg^{++} and the complement components have been collectively designated as the "properdin system" (227), a natural defense mechanism of blood. According to Pillemer this system kills certain bacteria (234), neutralizes some viruses (231, 11, 245) and lyses certain abnormal erythrocytes in the absence of specific antibodies (227, 237). A possible role of the properdin system in cancer immunity (233) has been indicated.

Properdin dissociated from PZ, when added to RP, will restore the various activities attributed to the properdin system. Efforts have also been made to isolate properdin from serum by fractionation with ethanol (244).

Administration of zymosan to mice produces a decline of properdin within one or two hours, followed by a rise within a few days to two weeks (228). Increase of properdin level has also been observed after injection of bacterial lipopolysaccharides (229) or polysaccharides from mammalian tissues (230). These polysaccharides combine with properdin and inactivate C′3.

Rabbit antisera have been prepared to human properdin and these sera will inhibit the various activities of properdin (232). According to Muschel *et al.* (247), these sera contain antibody to lysozyme.

Properdin may be assayed in terms of its capacity to inactivate C′3 in conjunction with zymosan and several procedures of this type have been described (110, 111, 240, 114). Another analytical approach is based on inactivation of phage by properdin (11). These different assays have not yielded uniformly concordant results (114). It was shown by Cowan (246) that there is no correlation between the activities of different human sera against T6 and T7 coliphages.

Detailed quantitative and kinetic studies of the reactions of zymosan or dextran with properdin and C′3 have been made by Leon (240-243). In addition to R3, Leon has used EAC′1, 4, 2 as a substrate for C′3 assay, but in view of the dual nature of C′3 (90) and the unresolved status of R3 in this respect, the interpretation of results may be uncertain. It is of interest that formation of PZ shows a lag period suggesting that this process may comprise more than one reaction step (242). Furthermore, Leon has shown that PZ decays at a rate proportional to the temperature (243), a process resembling the decay of EAC′1, 4, 2, and according to Leon PZ inactivates C′3 in the absence of divalent cations (243), like the reaction between EAC′1, 4, 2 and C′3.

A crucial issue in the properdin problem is the question whether the activities attributed to properdin might be due to natural antibodies. Could it be that the basis for distinction between properdin and antibody, as put forward by Pillemer, rests on experimental artifact or misinterpretation?

Nelson (88) has made the proposal that Pillemer's PZ complex is composed of zymosan, natural antibody to this carbohydrate and complement components C′1, C′4 and C′2, i.e., ZAC′1, 4, 2, in analogy with EAC′1, 4, 2. At 37°C. this intermediate product can react with C′3 in the presence of EDTA in a manner analogous to the reaction of EAC′1, 4, 2 with C′3. Furthermore, ZAC′1, 4, 2 undergoes decay to ZAC′1, 4, just like EAC′1, 4, 2 decays to EAC′1, 4. Hence, the requirement for complement and Mg^{++} in the second reaction step of the properdin system, i.e., inactivation of C′3 by PZ could be explained by assuming that ZAC′1, 4, 2 must be regenerated from ZAC′1, 4 by C′2 and Mg^{++}. In addition, Leon has demonstrated that fresh PZ, i.e., ZAC′1, 4, 2 in Nelson's view, will

react with C′3 in the presence of EDTA, again like the behavior of EAC′1, 4, 2.

It is Nelson's concept, then, that inactivation of C′3 by zymosan is a classical complement fixation reaction. In experiments with several guinea pig and human sera, he showed extensive fixation of C′1, 4, as well as C′3, by five different batches of zymosan. When expressed in terms of units of hemolytic activity, there was significantly more fixation of C′1, 4 than of C′3. Moreover, Nelson, as well as Osler *et al.* (12), have shown that at 37°C., C′3 is fixed by antigen-antibody complexes, in contrast to Pillemer's findings (87). Under the experimental conditions prevailing in the properdin experiments, small quantities of antigen-antibody complex, such as bovine plasma albumin and the corresponding rabbit antibody, may mimic the behavior of the properdin reaction in respect to the fact that C′3 is fixed extensively, while fixation of C′1, 4 remains relatively low when expressed as a percentage of the original number of units in fresh serum (88, 12). Thus, the primary definition of properdin and an important basis of distinction from antibody are questionable. Nelson (88) postulates that human serum contains traces of anti-carbohydrate capable of cross reacting with zymosan and that these antibodies give rise to the phenomena attributed by Pillemer to properdin. Nelson (88), as well as Blattberg (236) have also shown that guinea pigs and rabbits inoculated with zymosan form antibody detectable by agglutination and nitrogen uptake measurements. Purified properdin contains antibody to zymosan (88), and lysozyme also has been shown to be present (237).

However, if properdin is assumed to be antibody, or a collection of antibodies to various carbohydrate antigens, the question arises as to why its absorption from serum by zymosan appears to require complement. Perhaps, as suggested by Cowan (246), the findings of Maurer *et al.* (40, 41) provide an answer in the sense that readily dissociable antigen-antibody complexes may be stabilized by incorporation of complement.

With respect to the non-specific bactericidal action of properdin, as claimed by Pillemer (227), it has been found by Osawa and Muschel (248) that the bactericidal action of normal serum or RP against a "properdin-sensitive" strain of *Shigella dysenteriae* requires a specific substance which may be regarded as antibody in the classical sense. The studies of Muschel *et al.* (213) on the bactericidal action of normal serum against several Salmonella organisms also point to this conclusion. Recent studies of lysozyme (247) also implicate this agent in the bactericidal activities attributed to properdin (cf. 215).

Since the various activities attributed to properdin are dependent on complement, and in view of the fact that not all of the five complement components are available in purified form at present, it is difficult, and perhaps impossible to design and execute definitive experiments susceptible to clear-cut interpretation and yielding a definitive understanding of the nature and mode of action of the substance or substances called properdin. From the viewpoint of the student of complement, properdin can be regarded as a sensitizing agent, whether or not it turns out to be identical with natural antibodies. Definitive studies of the sensitizing agents present in serum will become possible only when the components of the complement system are available in highly purified form. The evidence at hand at present indicates that properdin may be an artifact and that its alleged activities may be due to natural antibodies and possibly lysozyme.

Immune-Adherence Reactions

A role has been ascribed to complement in the attachment of a variety of sensitized particulate antigens to erythrocytes or other "indicator particles" (2). The recent

work of Nelson and Nelson (8, 9) has clarified some of the general mechanisms involved in these reactions. For the present, these investigators restrict the term immune-adherence to the temperature-dependent attachment of complexes containing antigen, antibody, and complement to a receptor located on the surface of primate erythrocytes and on the surface of certain non-primate species of platelets. The reaction may be measured either by direct microscopic observation of the attached particulate antigen or by hemagglutination induced by soluble antigens in the presence of antibody and complement. Nelson and Nelson (8, 9) differentiate immune-adherence from the union of sensitized antigens to red cells from any species due to electrostatic bonding, termed "acid-adhesion," and from the agglutination of two unrelated sensitized antigens by complement, termed "complement-dependent mixed aggregation."

The nature of the receptor on the primate erythrocyte is not known but is assumed to be protein because of its inactivation by trypsin, chymotrypsin, papain and formaldehyde.

Immune - adherence requires, and is markedly sensitive to the presence of complement. For example, 1 ml. of fresh human serum diluted about 1/400, provides adequate complement for a variety of antigen-antibody systems. Reproducible reactivity at such high dilutions of human serum provides a latitude for measurement of decline of complement in disease states which is not attainable with the hemolytic assay with sheep erythrocytes and hemolysin. Similarly, the reaction is sensitive to small amounts of antibody, 0.005 to to 0.01 μg of antibody nitrogen being sufficient in the presence of a moderate excess of complement.

Evidence has been obtained that immune-adherence of staphylococci occurs *in vivo* in monkeys (3) and that the attachment of certain particulate antigens to erythrocytes leads to an increased ease of phagocytosis of the particles by normal leucocytes (1, 3). These observations suggest another possible mechanism whereby complement functions in host defense against pathogenic agents, particularly with reference to those organisms which are not susceptible to the bacteriolytic or bactericidal action of complement.

CHANGES OF COMPLEMENT IN DISEASE

Significant changes of complement activity have been observed in a wide variety of diseases, and following certain experimental treatments of animals. For example, changes in hemolytic complement activity have been used to study antigen-antibody reactions *in vivo* (249, 250). In experimental serum sickness, the complement titer declines during the phase of immune elimination of antigen (251, 252). However, development of lesions could not be related directly to decline in complement activity (253).

In acute glomerulonephritis (254-257) and in systemic lupus erythematosus (255, 258) hemolytic complement activity is usually low, and this may be considered presumptive evidence of antigen-antibody interaction *in vivo*, but other possible mechanisms have not been ruled out. On the other hand, in rheumatic fever (259-261) rheumatoid arthritis (255), myocardial infarction (262) and in various acute infections (19), human complement activity is frequently elevated above the normal level of 38 ± 4 $C'H_{50}$ (259). According to Fischel *et al.* (260, 262), this is another one of the "acute phase" phenomena accompanying wide-spread inflammatory processes. Sometimes, in conditions where a complement decline is expected, as in nephritis, a complicating infection or illness will cause an increase (254).

It is obvious that a decline of comple-

ment titer in disease could result from at least four causes, namely: 1) complement fixation by antigen-antibody complexes or aggregated gamma globulin, 2) decreased formation of one or more of the components, 3) increased breakdown or loss, or 4) formation of an inhibitor. Similarly, an increase in complement activity could result from several causes. For proper interpretation, it will be necessary to discriminate among the possible causes of change, and this will become possible only when acceptable methods for measuring individual complement components become available.

For further information on human complement, refer to Leon (235, 263-266).

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